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Interaction between the photoprotective protein LHCSR3 and C_2S_2 Photosystem II supercomplex in *Chlamydomonas reinhardtii*

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ABSTRACT

Photosynthetic organisms can thermally dissipate excess of absorbed energy in high-light conditions in a process known as non-photochemical quenching (NPQ). In the green alga *Chlamydomonas reinhardtii* this process depends on the presence of the light-harvesting protein LHCSR3, which is only expressed in high light. LHCSR3 has been shown to act as a quencher when associated with the Photosystem II supercomplex and to respond to pH changes, but the mechanism of quenching has not been elucidated yet. In this work we have studied the interaction between LHCSR3 and Photosystem II C_2S_2 supercomplexes by single particle electron microscopy. It was found that LHCSR3 predominantly binds at three different positions and that the CP26 subunit and the LHCII trimer of C_2S_2 supercomplexes are involved in binding, while we could not find evidences for a direct association of LHCSR3 with the PSII core. At all three locations LHCSR3 is present almost exclusively as a dimer.

1. Introduction

Oxygenic photosynthetic organisms harvest light via two large pigment-protein complexes called photosystem I (PSI) and II (PSII). The two photosystems, connected through the cytochrome b_6f complex, work in series to transform sun light into chemical energy. PSII is universally distributed throughout prokaryotes and eukaryotes capable of oxygenic photosynthesis, including higher plants, macroalgae and the oxyphotobacteria (cyanobacteria and prochlorophytes). In plants and algae a variable number of peripheral antenna proteins, belonging to the light-harvesting complex (LHC) multigenic family [1], can associate with dimeric PSII core complexes to form PSII-LHCII supercomplexes [2]. These supercomplexes have been denoted according to their composition. A dimeric core, C_2, can associate with up to six copies of peripheral LHCII trimers. Connection of the first two LHCII S trimers extends a C_2 complex to a C_2S_2 supercomplex, and two further M-trimers are bound in a $C_2S_2M_2$ supercomplex. In *Arabidopsis*, the $C_2S_2M_2$ supercomplex is the most abundant PSII particle under low light conditions [3]. In some species, such as *Chlamodomonas reinhardtii*, supercomplexes may bind a third type of trimer (called N), resulting in $C_2S_2M_2N_2$ supercomplexes [4,5], but these supercomplexes are less common than the smaller ones. A high-resolution three-dimensional structure of the spinach C_2S_2 supercomplex was recently determined using single-particle cryo-electron microscopy [6].

Photosynthetic organisms have developed mechanisms that can thermally dissipate excess of absorbed light in high-light conditions. This is necessary to prevent photodamage caused by reactive oxygen species produced by the reaction of chlorophyll triplet states with molecular oxygen [7]. The most rapid response to high light stress is a process known as non-photochemical quenching (NPQ) [8,9]. Excited chlorophylls in the antenna proteins become quenched and dissipate excess of absorbed energy as heat. The quenching involves the switch of the antenna proteins between a light-harvesting conformation, characterized by a long excited-state lifetime, and a quenched conformation that shows a shorter lifetime. The quenching mechanism is still under debate. It is known that NPQ is triggered by low lumenal pH, which is a signal for the overexcitation of the membrane [8,10]. The pH sensor for NPQ in plants is the PsbS protein [11,12]. In the green alga *C. reinhardtii* and the moss *Physcomitrella patens* different light-harvesting complex-like gene products, called LHCSR, have been found to be essential [13,14]. In *C. reinhardtii* the main responsible protein for NPQ is LHCSR3 [13], but LHCSR1 was recently shown to have similar properties [15]. LHCSR3 is a chlorophyll a/b and xanthophyll-binding protein of 253 amino acid residues [16] and has a folding very similar to that of LHCII [17]. The C-terminus of this protein was shown to contain a high number of protonable residues and to be responsible for the pH sensing [18].

It was reported that the PSII-LHCSR3 supercomplex formed in the high-light grown *C. reinhardtii* cells is capable of energy dissipation

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upon protonation of LHCSR3 [19]. An open question related to the function of LHCSR3 is where this protein interacts with PSII supercomplexes. The antenna protein LHCBM1 was shown to be involved in the quenching [20] and proposed to act as a partner of LHCSR3 [21]. More recently it has been suggested that LHCSR3 can bind to the core part of PSII supercomplexes, because evidence was found that in high-light acclimated *C. reinhardtii* the PSII subunit PsbR is crucial for the binding of LHCSR3 as well as for efficient NPQ [22]. PsbR is a 10-kDa protein that is involved in binding PsbP and is essential for optimal oxygen-evolving activity of PSII [23]. Its position in the core part is not yet clear because in the recently determined high-resolution structure of spinach C_2S_2 the oxygen-evolving complex subunits PsbO, PsbP and PsbQ are present, but PsbR is lacking [6].

To get information about the binding side of LHCSR3 in *C. reinhardtii* PSII, we applied single particle electron microscopy on a large data set of purified PSII-LHCSR3 complexes. It was found that LHCSR3 predominantly binds at three positions on the antenna part of C_2S_2 supercomplexes, but not at the core. Further, bound LHCSR3 is almost exclusively present as a dimer.

2. Materials & methods

2.1. Sample preparation

PSII supercomplexes were isolated from wild-type cells (137c) and *npq4* mutant *C. reinhardtii*. Cells were cultured in minimal medium (HSM) at room temperature (25 °C) a rotary shaker under continuous illumination of 500 μmol photons PAR m^2 s^{-1} ('high light') and 20 μmol photons PAR m^2 s^{-1} (normal light) collected in their exponential growth phase. Thylakoids were prepared as described [24]. For the purification of the supercomplexes [5], thylakoids were pelleted, unstacked with 5 mM EDTA and washed with 10 mM Hepes (pH 7.5). Membranes were then resuspended in 10 mM Hepes (pH 7.5), 0.15 M NaCl and solubilized at a final chlorophyll concentration of 0.5 mg/ml by adding an equal volume of 1.2% α-dodecyl maltoside (α-DM). Unsolubilized material was removed by centrifugation (12,000 rpm for 10 min at 4 °C). The supernatant was loaded on a 0–1.0 M sucrose gradient (+1 ml of 2 M cushion at the bottom of the gradient) and centrifuged at 280,000*g* for 14 h, 4 °C.

The green bands visible on the sucrose gradient were harvested with a syringe. The protein content of the bands was analyzed by SDS-6M urea PAGE with Tris-tricine buffer [25] and stained with Coomassie brilliant blue. Immunoblots were performed as in [26] using antibodies designed to specifically recognize LHCSR3 of *C. reinhardtii* (Agrisera). The PSII: LHCSR3 stoichiometry was determined by immunoblots. The LHCSR3 aproprotein overexpressed in *E. coli* [18] was quantified using bicinchoninic acid assay (Sigma) and used for the calibration curve. The absorption spectra were measured with a Varian Cary 4000 UV-Vis spectrophotometer in the buffer used for the sucrose gradient [27].

2.2. Electron microscopy and image analysis

Aliquots of 4 μl of samples of purified wild-type *C. reinhardtii* PSII-LHCSR3 particles were negatively stained with 2% uranyl acetate on glow discharged carbon-coated copper grids. Electron microscopy was performed on a Philips CM120 electron microscope operating at 120 kV. Images were recorded with a Gatan 4000 SP 4 K slow-scan CCD camera at 130,000× magnification at a pixel size of 0.225 nm at the specimen level with GRACE software for semi-automated specimen selection and data acquisition [28]. Single particle analysis was performed using GRIP software including multi-reference and non-reference alignments, multivariate statistical analysis and classification [29].

For the *C. reinhardtii npq4* mutant, sample preparation was performed in the same way as for wild-type particles. Imaging was performed on a Tecnai T20 at 200 kV and data acquisition was done as for wild-type particles, but at 133,000× magnification compatible to a pixel size of 0.224 nm. Single particles were analyzed with XMIP software (including alignments, statistical analysis and classification, as in [30] and RELION software [31].

3. Results

LHCSR3 is (almost) absent if *C. reinhardtii* cells are grown in low light (LL) conditions, but is induced after exposure (several hours) to high light stress [13,32,33]. To induce the expression of LHCSR3, *C. reinhardtii* cells were then grown in minimal medium in high light. As a first control the *npq4* mutant of *C. reinhardtii*, which does not express LHCSR3 [13], was grown in the same conditions and WT and as a second control the *npq4* mutant was grown in normal light. Immunoblotting analysis on the thylakoids purified from those cells (Fig. 1) confirms the presence of LHCSR3 only in the WT cells and especially in the cells grown in high light.

We then proceed to purify the PSII supercomplexes from WT and *npq4* cells grown in high light. Upon solubilization of the thylakoids and separation by sucrose density gradients centrifugations, 9 bands were observed (Fig. 2a), similarly to what we have obtained before for WT cells grown in normal light [5]. Based on the mobility in the gradient, the protein composition (Fig. 2b) and the absorption spectrum (Fig. 2c), the B7 fraction is expected to contain the C_2S_2 supercomplexes. As it was previously shown that LHCSR3 is associated with PSII supercomplexes [19], we checked by immunoblot the presence of LHCSR3 in the B7 fraction using an antibody that specifically recognizes LHCSR3 of *C. reinhardtii*. The results confirm the presence of this protein in the C_2S_2 fraction (Fig. 3) To determine the stoichiometry LHCSR3:PSII, we constructed a calibration curve by loading on the gel known amounts of the LHCSR3 aproprotein overexpressed in *E. coli* (Fig. 3). The loading of B7 was instead based on the Chl content. Because the Chl: protein stoichiometry for the C_2S_2 complex is known [6], the LHCSR3: PSII ratio could be calculated. The results indicate on average the presence of around 1 LHCSR3 on every 3 PSII supercomplexes

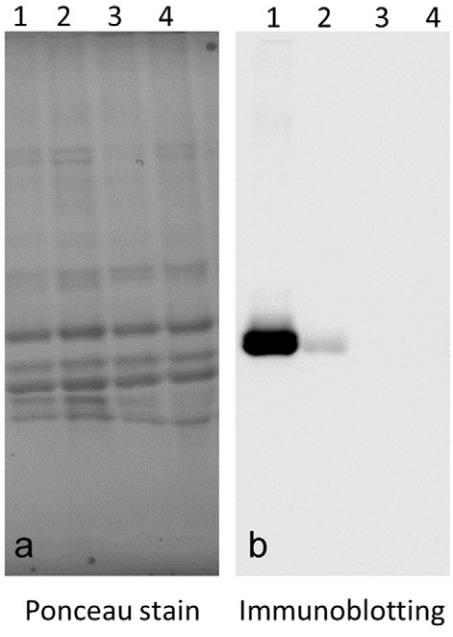

Fig. 1. Expression of LHCSR3 in different growth conditions. The presence of LHCSR3 in WT (1 and 2) and npq4 thylakoids (3 and 4) from cells grown in high light (1 and 3) and normal light (2 and 4) was determined by immunoblotting. (a) Ponceau staining (b) Immunoblot with antibodies against LHCSR3. The same amount of Chl (2 μg) was loaded in all lines.

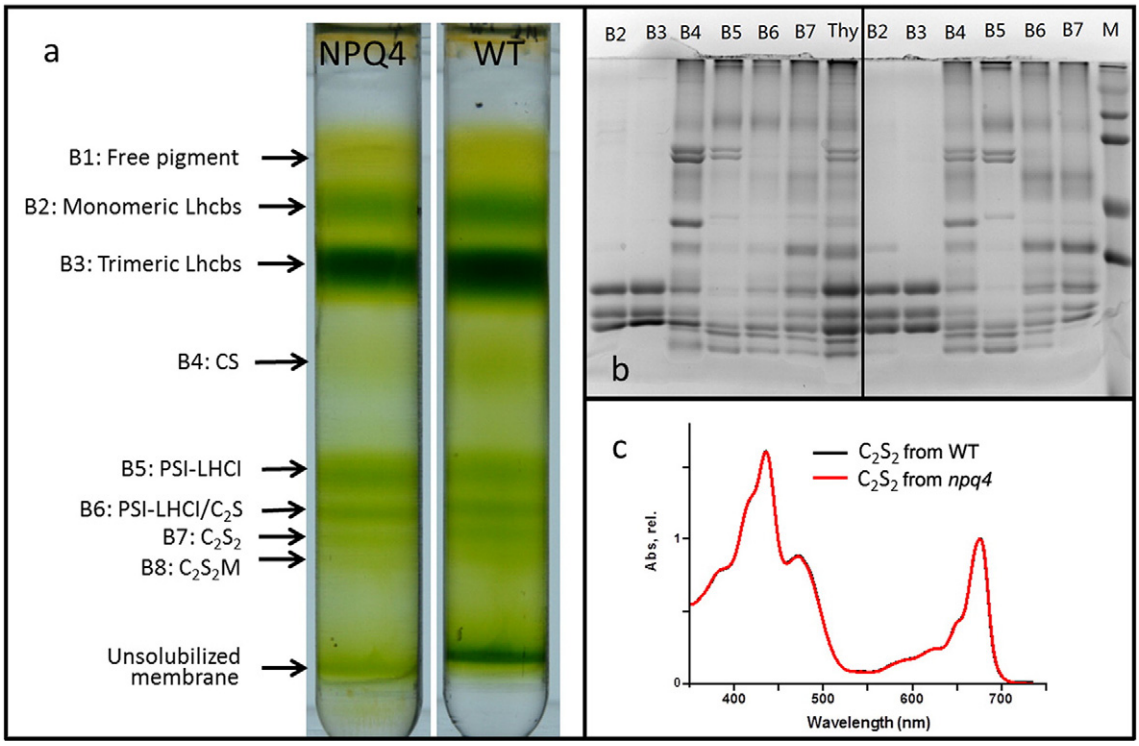

Fig. 2. Purification of the PSII (super)complexes. (a) Sucrose density gradients loaded with solubilized thylakoid membranes of *npq4* and WT grown in HL. The composition of the green bands is reported on the left. (b) SDS-PAGE showing the protein composition of each band (B2–B7) from WT (left) and npq4 (right) gradients. Thylakoid (Thy) and markers (M) are used as references. For a full characterization of the bands and the identification of the proteins see (Drop et al. 2014) (c) Absorption spectra of the B7 bands from WT (black) and npq4 (red) containing C2S2 complexes.

(0.31 ± 0.08), similarly to what observed before [19]. This means that LHCR3 should be present in a substantial number of particles and in principle enough to detect its density by EM. It should be noted that the LHCSR3:PSII stoichiometry reported here only refers to purified complexes and cannot be extrapolated to the *in vivo* situation.

3.1. Structural analysis of PSII-LHCSR3 by electron microscopy

To study the interaction between LHCSR3 and PSII, we examined a large data set of PSII particles from the B7 fractions, which is expected to contain the C_2S_2 PSII supercomplex by electron microscopy, which is the most abundant supercomplex in *C. reinhardtii*. The largest particle present was indeed this supercomplex. Larger particles, composed of additional M- and N-trimers, observed in low light grown cells, were not found, in agreement with the proposal that in high light part of the antenna is detached from the supercomplexes [33]. A visual inspection showed that about 30% of the C_2S_2 particles had a protrusion attached and from 20,000 recorded EM images a total of about 10,000 projections were selected for single particle analysis. Several homogeneous classes of projections show a tentative LHCSR3 protein attached. Basically, a bound protrusion was found at three different positions. In the first position, it is attached at the tip of the S-trimer, in two slightly different ways (Fig. 4a, b). This position was found in the majority of particles with a protrusion (63%). A second position was at the base of the supercomplex (Fig. 4c), close to the S-trimer and CP26 (13%). A third main position is at the side of CP26 (Fig. 4d; 21% of the particles). There was not much binding found at other places of the C_2S_2 supercomplex, except for small numbers (1%) at a site next to the Cyt b559 subunits of the dimeric core part (Fig. 4e). From the surface of the protrusion it seems that all main binding sites are occupied by two copies of LHCSR3 (see below). Analysis also indicated the presence of small numbers of PSII complexes with a smaller protrusion, of half the size of those of Fig. 4a–e. These smaller protrusions were seen in two positions (Fig. 4f,g) and likely represent a single LHCSR3 copy. All particles with a protrusion were distinctly different from those without (Fig. 3h).

3.2. Analysis of PSII from a npq4 mutant

To check the validity of the assignment of the PSII protrusions to LHCSR3, we performed single particle electron microscopy on a set of 150,000 projections of PSII particles (band B7), purified from the *npq4* mutant, which was also grown in HL but that does not express LHCSR3. The data set was classified and decomposed into 120 classes of which 100 showed recognizable maps with in total 110,000 particles in top view position. The largest group of classes (56% of the particles) showed the standard C_2S_2 supercomplex, of which the most detailed map is presented in Fig. 5a. No protrusions were visible in any of these 100 classes. The smaller C_2S particle, as shown in Fig. 5b, was present in 23% of the particles. Larger PSII supercomplexes were rare, but the C_2SMN supercomplex was present in one class, comprised of 2100 projections (Fig. 5c). About 14% of the complexes consisted of PSI complexes (Fig. 5d). Two classes with in total 1330 particles showed a potential protrusion similar to those assigned to LHCSR3 (Fig. 5e). This group of particles was further analyzed and classified and the largest homogeneous group of 750 projections shows a map that can be fully assigned to a C_2S_2M supercomplex, with the M trimer in a position as previously found [5]. There was no indication that any of the remaining 580 particles had a protrusion as found in Fig. 4, which was attributed to LHCSR3. In other words, out of 150,000 analyzed projections, the potential number of C_2S_2 complexes with a LHCSR protrusion is totally insignificant. This is firm evidence that the observed protrusions in Fig. 4 are composed of LHCSR3. As an additional control, we checked the presence

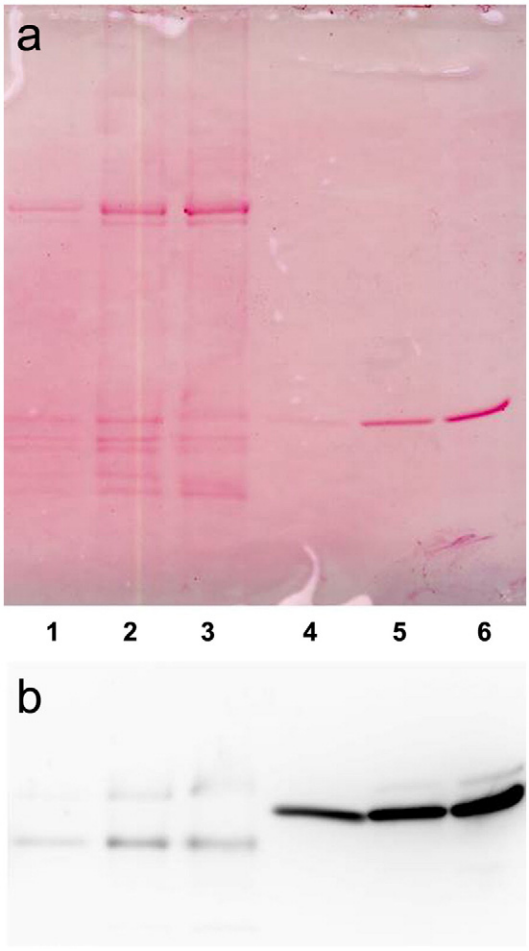

Fig. 3. LHCSR3: PSII stoichiometry. Different amounts of C2S2 supercomplexes (B7) are loaded in lines 1 (0.25 μg of Chl), 2 (1 μg) and 3 (0.5 μg). Lines 4, 5 and 6 are loaded with LHCSR3 0.25 μg, 0,5 μg and 1 μg of aproprotein, respectively. (a) Ponceau staining; (b) immunoblot with antibodies against LHCSR3. Please note that the difference in mobility of LHCSR3 is due to the His tag present on the recombinant protein.

of any protein density attached to C_2S_2 purified from cells grown in low light (20 μmol photons PAR m^{-2} s^{-1} [5], where LHCSR3 expression is low (Fig. 1). No particles with protrusions were found.

3.3. Structural modeling

To get a close impression of how LHCSR3 binds to C_2S_2 supercomplexes modeling was performed on some of the 2D projection maps. Modeling was applied for the three most abundant sites of dimeric interaction (Fig. 4a–c) and a monomeric interaction (Fig. 4g). We overlayed the projections with a dimeric core from the high-resolution plant PSII structure (yellow), trimeric LHCII (dark green) and monomeric CP26 and CP29 (bright green), in a similar but improved way as presented previously in the maps of *C. reinhardtii* PSII [5] and of *Arabidopsis* PSII, which has a highly homologous C_2S_2 PSII structure [3]. A LHCII monomer was used to fit LHCSR3 dimers [34]. The exact position of the dimers on PSII is ambiguous, because in projection the dimer is close to a rectangle. In principle, it could bind with its short or long side to PSII and because of lack of resolution the maps of Fig. 4a–e cannot give a clue. But because the low-abundant single LHCSR3 is attached with its short side to PSII (Fig. 4f, g), the most likely orientation of the dimers is as presented in Fig. 6. It should be noted, however, that another orientation of the monomers by a rotation of 180° within the dimer is equally possible.

4. Discussion

In the model green alga *C. reinhardtii* a specific type of LHC protein, called LHCSR3, is necessary for NPQ [13]. LHCSR3 was shown to bind to PSII [32] and to quench the PSII supercomplex at low pH [19]. In the work we have investigated the positions of the binding of LHCSR3. With this aim we have analysed a large set of PSII supercomplex particles containing LHCSR3. As a control we have analysed an even larger set of PSII particles purified from the *npq4* mutant, which does not express LHCSR3. The modelling presented in Fig. 6 shows the attachment of a LHCSR3 dimer in multiple positions to C_2S_2 PSII supercomplexes. The LHCSR3 protein was modeled as a dimer from the homologous LHCII protein of the Lhc protein family. This is in agreement with previous results indicating a dimeric state of LHCSR3 [16]. In the map of Fig. 6a, where the highest number of particles was averaged, the protrusion has the best defined shape and the LHCSR3 dimer fits well within its surface. Another observation from Fig. 6a–c is the fact that there is no negative stain intercalating between the protrusion and the PSII supercomplex. This is indicative for a real association, instead of an aggregation of LHCSR3 dimers and PSII supercomplexes after solubilization. A typical example of such an aggregation after purification are sandwiched PSII supercomplexes, which are always recognizable by a thick layer of negative stain between the two supercomplexes [35]. The fact that about 30% of all the observed PSII complexes has the distinct protrusion, assigned to LHCSR3, is compatible with the presence of this protein in the preparation. In this respect it is important to realize that the LHCSR3: PSII stoichiometry obtained by EM cannot be exactly compared with the data obtained biochemically. This because during single particle analysis a subset of the most intact particles, which apparently contain LHCSR3, is selected, introducing a positive bias, while the biochemical data represent an average. A more direct assignment of the protrusion with for instance antibody labeling was not possible. But the absence of any protein protrusion attached to C_2S_2 purified from (1) cells grown in low light [5], where LHCSR3 expression is low and (2) from 150,000 images of (possible) PSII supercomplexes of the *npq4* mutant grown in high light (Fig. 4) is strong evidence for the assignment of the protrusions to LHCSR3.

There have been two previous suggestions for the binding position of LHCSR3. First, the antenna protein LHCBM1 was proposed to act as a partner of LHCSR3 [21]. It is however not known where the LHCBM1 protein is located in the PSII supercomplex. It can be a regular component of either the S-trimer or the M-trimer or both [5] and therefore no firm conclusions about the LHCSR3 position could be derived. More recently it has been suggested that LHCSR3 can bind to the core part of PSII supercomplexes [22], because evidence was found that in high-light acclimated *C. reinhardtii* the PSII subunit PsbR is crucial for the binding of LHCSR3. In contrast, our data show clear binding positions of LHCSR3 at the S-trimer of PSII C_2S_2 supercomplex, but no substantial binding to the core. We cannot exclude that additional binding sites for LHCSR3 are present *in vivo*, but is this is the case the association of LHCSR3 with them should be very loose as it does not survive purification. Alternatively, it is thus possible that the absence of PsbR influences the binding of some antenna and as a result that of LHCSR3.

Although in *C. reinhardtii* LHCSR3 is the most important protein responsible for quenching, recently a role for PsbS and LHCSR1 [36] have also emerged. Although PsbS cannot compensate for the function of LHCSR3 in an LHCSR-deficient mutant, it accumulates very rapidly and transiently during high light stress [37] and is essential for the activation of non-photochemical quenching in *C. reinhardtii* [37,38]. It is therefore important to incorporate PsbS and LHCSR1 in future studies about the function of LHCSR3 in green algae.

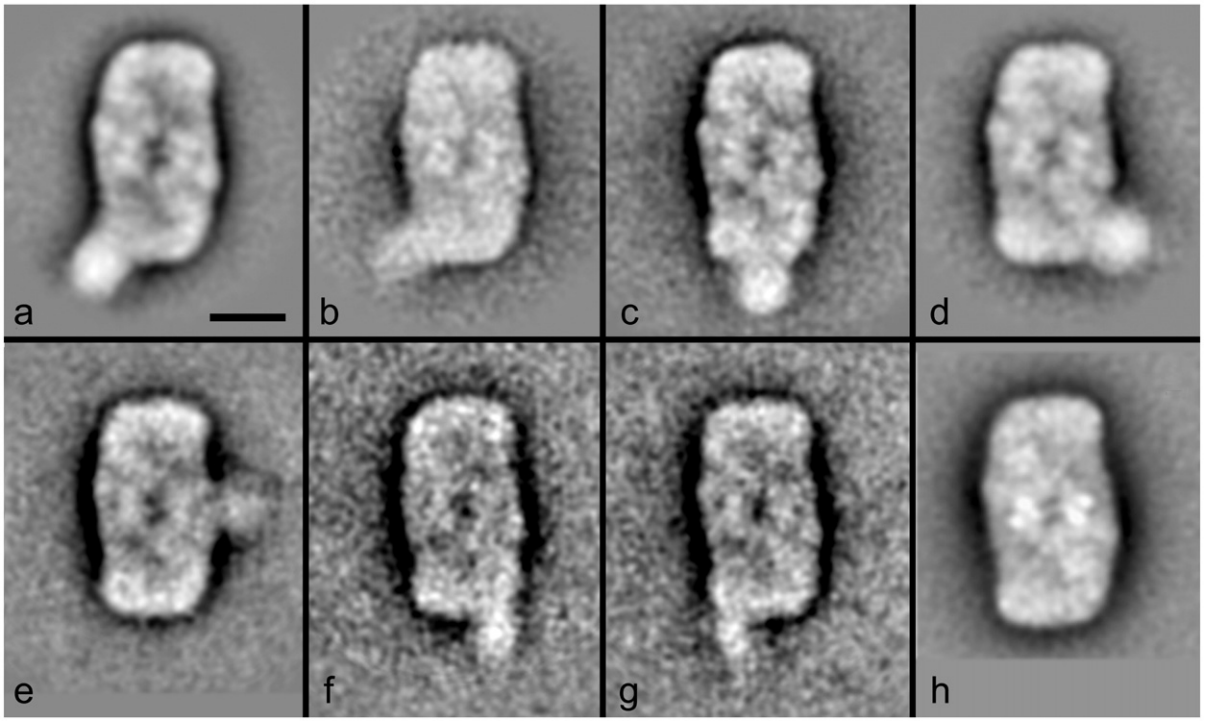

Fig. 4. EM maps of *Chlamydomonas* PSII-LHCSR3 complexes obtained by single particle averaging. (a–e) C_2S_2 particles with a LHCSR3 dimer attached in 5 different positions. Sums of 2929, 197, 588, 1392, 138, 33 and 42 projections, respectively. (f, g) C_2S_2 particles with a LHCSR3 monomer attached. (h) C_2S_2 particle without LHCSR3, sum of ~1000 projections. Scale bar for all frames equals 100 Å.

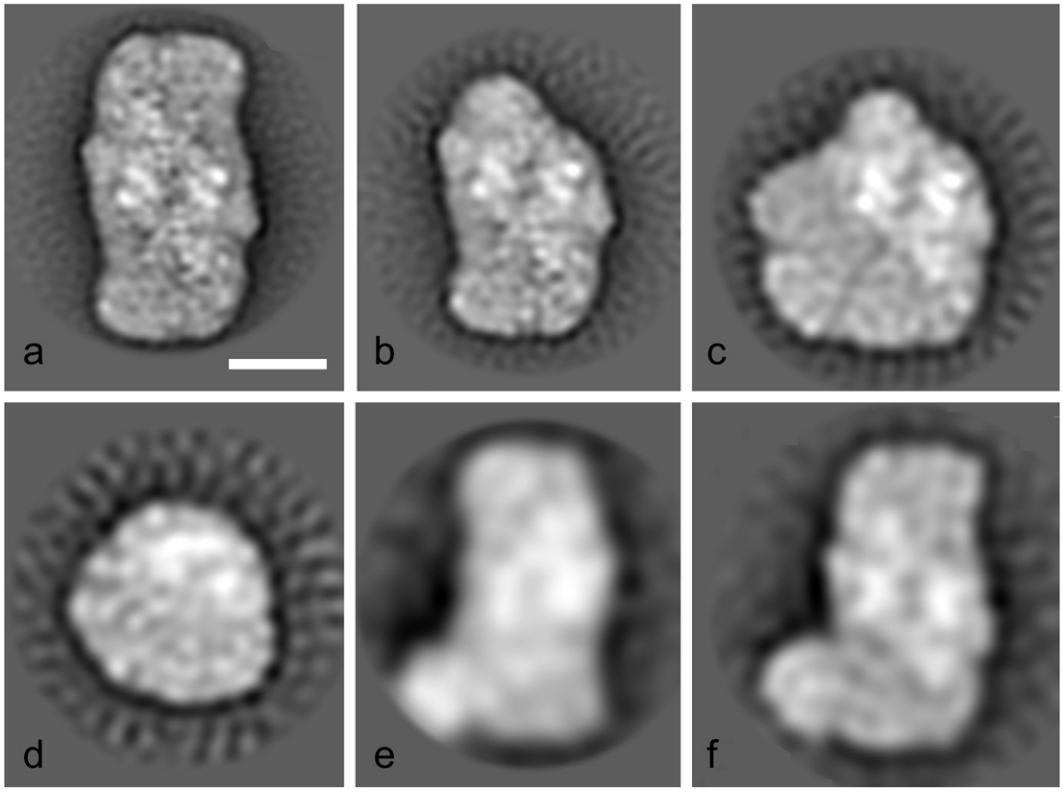

Fig. 5. Analysis of PSII complexes from a *npq4* mutant. (a) Best class of C_2S_2 particle, sum of 11,200 projections. (b) Best C_2S supercomplex class, sum 6200 projections. (c) C_2SML supercomplex, sum of 2100 projections. (d) Best PSI class, sum of 2700 projections. (e) Pool of two classes of 1400 C_2S_2 supercomplexes projections, with a potential protrusion. (f) Class of 450 C_2S_2M particles from re-analyzed particles of the pool of 1400 C_2S_2 particles. The scale bar for all frames is 100 Å.

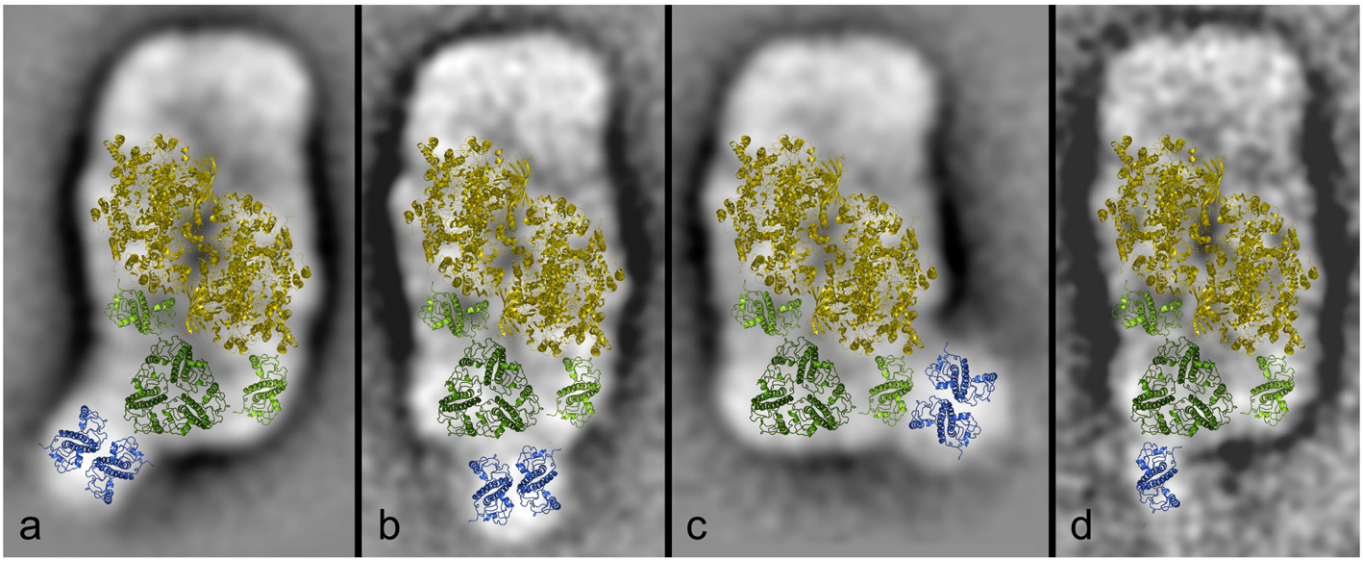

Fig. 6. Modeling of the binding of LHCSR3 dimers or monomers on C_2S_2 PSII supercomplexes. Structures of dimeric PSII core (yellow), S trimer (dark green), CP26 and CP29 (bright green) [6] and LHCSR3 (blue), modelled from a LHCII monomer of LHCII, [34] are indicated.

Conflict of interests

- No conflicts of interest.
- No matters concerning thirds parties.
- No matters concerning patents or whatsover.

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